Published online September 8, 2006

Effects of Xenia on *Aspergillus flavus* Infection and Aflatoxin Accumulation in Maize Inbreds

Hilarie D. Gardner,* W. Paul Williams, and Gary L. Windham

ABSTRACT

***Aspergillus flavus* Link:Fries infection and aflatoxin contamination pose an economic threat to maize (*Zea mays* L.) producers of the United States. Efforts to identify germplasm resistant to *A. flavus* infection and aflatoxin accumulation have raised questions regarding the role of xenia, the pollen effect on the embryo and endosperm, in resistance of maize grain to the pathogen. The objective of this study was to evaluate the importance of xenia on *A. flavus* infection and aflatoxin accumulation in seed of eight inbred lines with different levels of resistance to *A. flavus* infection and aflatoxin contamination. Resistant and susceptible maize lines were hand-pollinated following a diallel mating design to produce seed for trials. The ears were inoculated 14 d after pollination with *A. flavus* spores. Grain was plated on agar to determine the extent of *A. flavus* infection and analyzed to measure aflatoxin content. Significant differences were detected among seed parents for both aflatoxin accumulation and *A. flavus* infection in both 2003 and 2004. The effects of pollen source were not significant on aflatoxin contamination or *A. flavus* infection in either 2003 or 2004. These results are consistent with xenia having little or no effect on *A. flavus* infection or aflatoxin accumulation. The results further suggest that reliable evaluation of *A. flavus* infection and aflatoxin contamination can be gained from open-pollinated field experiments.**

Since its discovery in 1960 as the causal agent of turkey X disease, aflatoxin, produced by the fungus *A. flavus*, has been shown to be linked to numerous human and animal diseases (Bennett and Klich, 2003; Gourama and Bullerman, 1995). Due to the ill effects stemming from inadvertent inhalation or ingestion of this toxin, contamination of maize grain with aflatoxin ranks among the primary concerns of maize producers in the Midwest and southeastern USA today. Because aflatoxin is a known carcinogen, stringent regulations have been put into effect by the U.S. Food and Drug Administration. Aflatoxin B_1 is considered the most toxic compound of the aflatoxin family. Currently, grain having a concentration of aflatoxin B_1 exceeding the threshold of 20 ng g^{-1} is banned from interstate trade (U.S. Food and Drug Administration, 1992). Enforcement of these restrictions has caused substantial economic losses to maize growers of the southeast and midwest regions of the United States. As a result, maize breeders and researchers have been attempting to discover factors influencing aflatoxin production by *A. flavus* to determine an effective means to reduce or eliminate aflatoxin production in grain.

Climatic conditions in the southern regions of the USA tend to favor preharvest contamination of maize grain with aflatoxin (Lillehoj et al., 1975; Zuber et al., 1976). Numerous studies have likewise implicated heat, water, nutrient, and insect stress in providing favorable conditions for *A. flavus* infection and subsequent aflatoxin accumulation (Jones and Duncan, 1981; Jones et al., 1981; McMillian et al., 1985; Widstrom et al., 1975, 1990). To combat aflatoxin contamination of maize grain, plant breeders and geneticists are currently screening germplasm and selecting maize lines with the goal of incorporating resistance traits into commercially grown hybrids (Tubajika and Damann, 2001; Zuber, 1977). Currently, many researchers are investigating proteins in various maize lines to identify those linked to inhibition of *A. flavus* infection and reduced aflatoxin accumulation. Proteins associated with antifungal properties (Chen et al., 2001; Guo et al., 1998; Nielsen et al., 2001) and insect defense mechanisms (Pechan et al., 2002; Rector et al., 2002; Williams et al., 2005) in maize plants have lately garnered interest, and more studies are being conducted on proteins present in the cob and kernel (Alfaro, 1999; Magbanua, 2004) that may be involved in *A. flavus* resistance.

Investigations of resistance to *A. flavus* infection and aflatoxin accumulation in maize grain are complex because the seed consists of genetically different tissues: the diploid embryo and triploid endosperm that results from double fertilization, as well as the maternally derived pericarp. Other maternally derived tissues of the ear such as the silks, husks, and cobs may also play a role in fungal establishment and toxin accumulation in the seed. Recent studies of xenia have focused on its impact on grain yield components such as kernel weight, moisture, and protein composition (Bulant and Gallais, 1998; Bulant et al., 2000; Seka and Cross, 1995; Seka et al., 1995; Tsai and Tsai, 1990; Weingartner et al., 2002). Researchers studying *A. flavus* infection and aflatoxin accumulation have generally based their observations on grain produced on open-pollinated ears, thus the role of xenia in *A. flavus* and aflatoxin resistance in the embryo and endosperm has yet to be clearly defined.

The objective of this investigation was to evaluate *A. flavus* infection and aflatoxin accumulation in grain produced on eight inbred lines that had been pollinated in all possible combinations. This differed from traditional diallels in that the analyses were conducted on grain containing the F_1 embryo that was produced on an inbred plant, rather than grain produced on an F_1 plant. The effects of each inbred line used as a pollen source

USDA-ARS, Corn Host Plant Resistance Research Unit, Box 9555, Mississippi State, MS 39762. This paper is a joint contribution of USDA-ARS and the Mississippi Agricultural and Forestry Experiment Station and is published as journal no. J-10790 of the Miss. Agric. and Forestry Exp. Stn. Received 28 Mar. 2006. *Corresponding author (hgardner@msa-msstate.ars.usda.gov).

Published in Crop Sci. 46:2151–2154 (2006).
Crop Breeding & Genetics—Notes
doi:10.2135/cropsci2006.02.0071

677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: GLM, General Linear Models; LSD, least significant difference; NRRL, Northern Regional Research Laboratory.

for the seed parents were compared to determine the relative importance of xenia on *A. flavus* infection and aflatoxin accumulation among this group of inbred lines. The information gained from these analyses should help to identify inbred lines that would be most useful in maize breeding programs targeted at reducing aflatoxin levels in grain and in determining whether xenia should be considered in germplasm evaluations.

MATERIALS AND METHODS

Resistant maize inbred lines, Mp313E, Mp420, Tx601, and Mo18W (Scott and Zummo, 1990, 1992; Williams et al., 2003; Windham and Williams, 2002), and susceptible inbred lines, SC212M, SC229, Ab24E, and Mp339 (Williams et al., 2003) were planted at the R.R. Foil Plant Science Research Farm, Mississippi State, Mississippi, on 17 Apr. 2003 and 20 Apr. 2004. Plots were single rows 4 m long and spaced 1 m apart. Plots were overplanted and thinned to 15 plants after seedling emergence. In 2003, plants were hand-pollinated to produce seed of all possible crosses, including reciprocals, among the inbred lines. In 2004, all possible crosses among the inbred lines were again made, but each inbred line was also self-pollinated. Because the inbred lines differed in maturity, additional rows of each line were planted in rows bordering the experimental trial in both 2003 and 2004. Plantings in these border rows were initiated 3 wk before the experiment was planted and continued for 5 wk. These additional rows were planted to provide a source of pollen for making crosses between genotypes differing in maturity. Planting all seed parents on the same day, but making multiple plantings of each inbred line to provide pollen, minimized the time required to complete pollinations of the seed parent. Each seed parent–pollen parent combination was assigned to a single-row plot. Plots were arranged in a randomized complete block design with four replications. In 2003, each replication included seven plots of each inbred line for a total of 56 plots per replication. The inbred line in each plot was hand-pollinated with pollen from the designated line. In 2004, one additional plot of each line was included and self-pollinated.

Twelve to fourteen days after hand-pollination, the primary ears of each maize plant were inoculated using the side-needle technique described by Zummo and Scott (1989) with *A. flavus* isolate NRRL 3357, an isolate known to produce aflatoxin in maize. Ears from each row (approximately 15 ears) were hand-harvested, bulked, and shelled. The grain was thoroughly mixed, and a 150-g sample was taken for aflatoxin analysis. The samples were ground using a Romer mill (Union, MO), and the concentration of aflatoxin was determined using Aflatest (Vicam, Watertown, MA) which can detect aflatoxin levels as low as 1 ng g^{-1}.

To determine the percentage of kernels infected by *A. flavus*, a sample of 130 kernels that exhibited no visual signs of damage was taken from each plot, surface sterilized, and plated on Czapek solution agar (29 g L^{-1}) with NaCl (75 g L^{-1}) (Zummo and Scott, 1992). After 7 d, the number of kernels with *A. flavus* colonies were counted and the percentage of kernels infected by the fungus determined.

The aflatoxin data were transformed as ln(y + 1), where y is equal to the concentration in ng g^{-1} of aflatoxin in a sample, for analysis of variance using the SAS General Linear Models (GLM) procedure. This transformation was performed to provide a more normally distributed set of data for statistical analysis. For both aflatoxin concentration and fungal infection data, the variation associated with genotypes, or seed parent–pollen parent combinations, was partitioned into components for seed parents, pollen parents, and the interaction between seed and pollen parents. Means for *A. flavus* infection and aflatoxin concentration in grain among seed and pollen parents were compared using Fisher's Protected Least Significance Difference (FLSD) at $P = 0.05$ level of significance. Different degrees of freedom for each year resulted from an inadequate production of grain for some crosses.

RESULTS AND DISCUSSION

The mean levels of aflatoxin contamination and *A. flavus* infection for each inbred line used as a seed parent and as a pollen source in 2003 are given in Table 1. Because sufficient grain was not produced in crosses with Ab24E, data for this line were not included in the statistical analysis. The analysis of variance indicated that seed parent was a significant source of variation for both aflatoxin contamination and *A. flavus* infection, but the variation associated with pollen source or the interaction of seed parents and pollen source was not statistically significant ($P = 0.05$).

Among the seed parents, SC212M exhibited the highest levels of aflatoxin contamination when averaged across pollen sources (2160 ng g^{-1}). Mp313E, which was developed and released as a source of resistance to *A. flavus* (Scott and Zummo, 1990), exhibited the lowest levels of aflatoxin contamination. The mean aflatoxin contamination level across pollen sources was 10 ng g^{-1}. Tx601 and Mp420, also aflatoxin-resistant lines, and SC229 had significantly less aflatoxin contamination than the more susceptible lines, but higher levels than Mp313E. The mean levels of aflatoxin contamination did not differ among the seven lines when used as sources of pollen.

As a seed parent, SC212M exhibited the highest percentage of *A. flavus*–infected kernels. Averaged across pollen sources, the mean level of infection for SC212M was 23%. SC212M also exhibited the highest level of aflatoxin contamination. Differences among the other lines as seed parents were not statistically significant. As sources

Table 1. Aflatoxin contamination and *A. flavus* infection of maize kernels produced on seven inbred lines hand-pollinated following a diallel mating design and inoculated with *A. flavus* in 2003.

	Aflatoxin mean†		*A. flavus* infection‡	
Inbred line	Seed parent§	Pollen source¶	Seed parent§	Pollen source¶
	ng g^{-1}		%	
SC212M	2160 a#	131	23 a††	3‡‡
Mp339	962 b	453	5 b	7
Mo18W	319 c	107	3 b	7
SC229	114 d	240	3 b	10
Mp420	102 d	149	3 b	6
Tx601	79 d	305	2 b	7
Mp313E	10 f	272	1 b	6

† Tests of significance were performed on transformed ln(y + 1) means before converting logarithmic means to the original scale.
‡ *A. flavus* kernel infection was determined by plating 130 kernels from each plot on Czapek solution agar and counting kernels with *A. flavus* colonies.
§ Each value represents the mean for all seed parent–pollen parent combinations for which the given inbred was the seed parent.
¶ Each value represents the mean for all seed parent–pollen parent combinations for which the given inbred was the source of pollen.
# Means in a column followed by the same letter do not differ at $P = 0.05$.
†† $F = 37.56$; df = 6, 70; $P < 0.001$.
‡‡ $F = 0.30$; df = 6, 70; $P = 0.936$.

of pollen, the lines did not differ in percentage of *A. flavus*–infected kernels.

In 2004, self-pollinated plants were also included in the field trials. Unfortunately, insufficient grain was produced by Mp313E and Mp420 for inclusion of these lines as seed parents in the statistical analyses. Because of these differences, the data from 2004 were not combined with the data from 2003 for statistical analysis. As in 2003, seed parent was a significant source of variation in the analysis of variance in 2004. The variance associated with neither source of pollen nor the interaction of seed parent and pollen source was statistically significant ($P = 0.05$).

Mp339, as a seed parent, exhibited the highest level of aflatoxin contamination (2677 ng g^{-1}) in 2004 (Table 2). SC212M and Mo18W had the second highest levels of aflatoxin in 2004. These three inbred lines also had the highest levels of aflatoxin in 2003. Tx601 and SC229, as seed parents, had the lowest levels of aflatoxin contamination in 2004. In 2003, only Mp313E had lower levels of aflatoxin contamination. As in 2003, there were no significant differences among the eight inbred lines when used as sources of pollen for either aflatoxin accumulation or percentage of *A. flavus*–infected kernels. Among the inbred lines used as seed parents in 2004, SC212M again had the highest level of *A. flavus*–infected kernels (33%). Mp420 exhibited the second highest percentage of kernel infection in 2004, and only Ab24E and Tx601 had significantly lower levels of kernel infection. In this investigation, the inbred lines with high percentages of *A. flavus*–infected kernels tended to have high levels of aflatoxin contamination as well.

The lack of statistically significant differences associated with source of pollen and the interaction between seed parents and pollen sources for both percentage of *A. flavus* infection and the concentration of aflatoxin contamination are consistent with a conclusion that xenia, the effect of the pollen on the embryo and endosperm, does not play a major role in the expression of these traits. It appears that evaluation for resistance or susceptibility to *A. flavus* infection and aflatoxin contamination can be achieved in open-pollinated field experiments conducted on maize so that the harvested grain is described solely in terms of the maternal parent. This would mitigate the need for controlled pollinations using a specific genotype as a source of pollen. Although dependable germplasm evaluations can probably be performed with open pollination, it would be imprudent to completely rule out xenia's influence in all types of experiments since this particular study was based on two environments only and a fixed set of lines. In those studies conducted to identify genes or proteins associated with resistance to aflatoxin accumulation or fungal infection, it would be desirable to define the genotype of both embryo and endosperm to avoid introducing unaccounted for genetic variability. The results of this investigation are also consistent with the proposal that maternal tissues such as pericarp, silk, husk, and cob, rather than the embryo or endosperm, may play key roles in determining the resistance or susceptibility among this group of inbred lines of maize (Guo et al., 1998; Rector et al., 2002).

Table 2. Aflatoxin contamination and *A. flavus* infection of maize kernels produced on six inbred lines hand-pollinated following a diallel mating design and inoculated with *A. flavus* in 2004.

	Aflatoxin mean†		*A. flavus* infection‡	
Inbred line	Seed parent§	Pollen source¶	Seed parent§	Pollen source¶
	ng g^{-1}		%	
Mp339	2677 a#	602	13 bc††	17‡‡
Mo18W	1013 b	384	12 bc	11
SC212M	905 b	644	33 a	12
Ab24E	508 c	717	6 c	12
SC229	152 d	426	14 bc	15
Tx601	126 d	473	6 c	14
Mp420	–	441	18 b	18
Mp313E	–	548	–	17

† Tests of significance were performed on transformed ln(y + 1) means before converting logarithmic means to the original scale.
‡ *A. flavus* kernel infection was determined by plating 130 kernels from each plot on Czapek solution agar and counting kernels with *A. flavus* colonies.
§ Each value represents the mean for all seed parent–pollen parent combinations for which the given inbred was the seed parent.
¶ Each value represents the mean for all seed parent–pollen parent combinations for which the given inbred was the source of pollen.
# Means in a column followed by the same letter do not differ at $P = 0.05$.
†† $F = 22.25$; df = 6, 115; $P < 0.0001$.
‡‡ $F = 0.50$; df = 7, 115; $P = 0.666$.

ACKNOWLEDGMENTS

The authors express their appreciation to Clarence E. Watson and Jixiang Wu who provided advice and statistical support for the experiments and to Ladonna Owens for her technical assistance. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

REFERENCES

Alfaro, Y. 1999. Response of resistant and susceptible maize genotypes to inoculation with transformed *Aspergillus flavus* isolates. Ph.D. diss. Mississippi State Univ., Mississippi State, MS.

Bennett, J.W., and M. Klich. 2003. Mycotoxins. Clin. Microbiol. Rev. 16:497–516.

Bulant, C., and A. Gallais. 1998. Xenia effects in maize with normal endosperm: I. Importance and stability. Crop Sci. 38:1517–1525.

Bulant, C., A. Gallais, E. Matthys-Rochon, and J.L. Prioul. 2000. Xenia effects in maize with normal endosperm: II. Kernel growth and enzyme activities during grain filling. Crop Sci. 40:182–189.

Chen, Z.Y., R.L. Brown, T.E. Cleveland, K.F. Damann, and J.S. Russin. 2001. Comparison of constitutive and inducible maize kernel proteins of genotypes resistant or susceptible to aflatoxin production. J. Food Prot. 64:1785–1792.

Gourama, H., and L.B. Bullerman. 1995. *Aspergillus flavus* and *Aspergillus parasiticus*: Aflatoxigenic fungi of concern in foods and feeds: A review. J. Food Prot. 58:1395–1404.

Guo, B.Z., R.L. Brown, A.R. Lax, T.E. Cleveland, J.S. Russin, and N.W. Widstrom. 1998. Protein profiles and antifungal activities of kernel extracts from corn genotypes resistant and susceptible to *Aspergillus flavus*. J. Food Prot. 61:98–102.

Jones, R.K., and H.E. Duncan. 1981. Effect of nitrogen fertilizer, planting date, and harvest date on aflatoxin production in corn inoculated with *Aspergillus flavus*. Plant Dis. 65:741–744.

Jones, R.K., H.E. Duncan, and P.B. Hamilton. 1981. Planting date, harvest date, and irrigation effects on infection and aflatoxin production by *Aspergillus flavus* in field corn. Phytopathology 71: 810–816.

Lillehoj, E.B., W.F. Kwolek, E.E. Vandegraft, M.S. Zuber, O.H. Calvert, N. Widstrom, M.C. Futrell, and A.J. Bockholt. 1975. Aflatoxin production in *Aspergillus flavus* inoculated ears of corn grown at diverse locations. Crop Sci. 15:267–270.

Magbanua, Z.V. 2004. *Zea mays* L. resistance to *Aspergillus flavus*: The path of infection and the role of catalase. Ph.D. diss. Mississippi State Univ., Mississippi State, MS.

McMillian, W.W., D.M. Wilson, and N.W. Widstrom. 1985. Aflatoxin contamination of preharvest corn in Georgia: A six-year study of insect damage and visible *Aspergillus flavus*. J. Environ. Qual. 14:200–202.

Nielsen, K., G.A. Payne, and R.S. Boston. 2001. Maize ribosome-inactivating protein inhibits normal development of *Aspergillus nidulans* and *Aspergillus flavus*. Mol. Plant Microbe Interact. 14: 164–172.

Pechan, T., A. Cohen, W.P. Williams, and D.S. Luthe. 2002. Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. Proc. Natl. Acad. Sci. USA 99: 13319–13323.

Rector, B.G., M.E. Snook, and N.W. Widstrom. 2002. Effect of husk characters on resistance to corn earworm (Lepidoptera: Noctuidae) in high-maysin maize populations. J. Econ. Entomol. 95:1303–1307.

Scott, G.E., and N. Zummo. 1990. Registration of Mp313E parental line of maize. Crop Sci. 30:1378.

Scott, G.E., and N. Zummo. 1992. Registration of Mp420 germplasm line of maize. Crop Sci. 32:1296.

Seka, D., and H.Z. Cross. 1995. Xenia and maternal effects on maize kernel development. Crop Sci. 35:80–85.

Seka, D., H.Z. Cross, and P.E. McClean. 1995. Maize kernel development in vitro: Sucrose concentration, xenia, and maternal effects. Crop Sci. 35:74–79.

Tsai, C.L., and C.Y. Tsai. 1990. Endosperm modified by cross-pollinating maize to induce changes in dry matter and nitrogen accumulation. Crop Sci. 30:804–808.

Tubajika, K.M., and K.E. Damann. 2001. Sources of resistance to aflatoxin production in maize. J. Agric. Food Chem. 49:2652–2656.

U.S. Food and Drug Administration. 1992. FDA regulatory guidance for toxins and contaminants [Online]. Available at www.ngfa.org/toxinsPDF-1.pdf (accessed 7 June 2005; verified 16 June 2006). Natl. Grain and Feed Assoc., Washington, DC.

Weingartner, U., O. Kaeser, M. Long, and P. Stamp. 2002. Combining cytoplasmic male sterility and xenia increases grain yield of maize hybrids. Crop Sci. 42:1848–1856.

Widstrom, N.W., W.W. McMillian, R.W. Beaver, and D.M. Wilson. 1990. Weather-associated changes in aflatoxin contamination of preharvest maize. J. Prod. Agric. 3:196–199.

Widstrom, N.W., A.N. Sparks, E.B. Lillehoj, and W.F. Kwolek. 1975. Aflatoxin production and lepidopteran insect injury on corn in Georgia. J. Econ. Entomol. 68:855–856.

Williams, W.P., G.L. Windham, and P.M. Buckley. 2003. Enhancing maize germplasm with resistance to aflatoxin contamination. J. Toxicol–Toxin Rev. 22:175–193.

Williams, W.P., G.L. Windham, P.M. Buckley, and J.M. Perkins. 2005. Southwestern corn borer damage and aflatoxin accumulation in conventional and transgenic corn hybrids. Field Crops Res. 91: 329–336.

Windham, G.L., and W.P. Williams. 2002. Evaluation of corn inbreds and advanced breeding lines for resistance to aflatoxin contamination in the field. Plant Dis. 86:232–234.

Zuber, M.S. 1977. Influence of plant genetics on toxin production in corn. p. 173–179. *In* J.V. Rodricks, D.W. Hesseltine, and M.A. Mehlman (ed.) Mycotoxins in human and animal health. Pathotox Publishers, Inc., Park Forest South, IL.

Zuber, M.S., O.H. Calvert, E.B. Lillehoj, and W.F. Kwolek. 1976. Preharvest development of aflatoxin B_1 in corn in the United States. Phytopathology 66:1120–1121.

Zummo, N., and G.E. Scott. 1989. Evaluation of field inoculation techniques for screening maize genotypes against kernel infection by *Aspergillus flavus* in Mississippi. Plant Dis. 73:313–316.

Zummo, N., and G.E. Scott. 1992. Interaction of *Fusarium moniliforme* and *Aspergillus flavus* on kernel infection and aflatoxin contamination in maize ears. Plant Dis. 76:771–777.